Journal of Radiation Research, Vol. 61, No. 1, 2020, pp. 68–72
doi: 10.1093/jrr/rrz074
Advance Access Publication: 11 December 2019

The use of a centrifuge-free RABiT-II system for high-throughput micronucleus analysis

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(Received 10 June 2019; revised 16 September 2019; editorial decision 26 September 2019)

ABSTRACT

The cytokinesis-block micronucleus (CBMN) assay is considered to be the most suitable biodosimetry method for automation. Previously, we automated this assay on a commercial robotic biotech high-throughput system (RABiT-II) adopting both a traditional and an accelerated micronucleus protocol, using centrifugation steps for both lymphocyte harvesting and washing, after whole blood culturing. Here we describe further development of our accelerated CBMN assay protocol for use on high-throughput/high content screening (HTS/HCS) robotic systems without a centrifuge. This opens the way for implementation of the CBMN assay on a wider range of commercial automated HTS/HCS systems and thus increases the potential capacity for dose estimates following a mass-casualty radiological event.

Keywords: micronucleus assay; automation; biodosimetry; high-throughput

INTRODUCTION

In the case of a mass-casualty radiation event, it is necessary to estimate radiation doses to hundreds of thousands of individuals [1]. On smaller scales this is done manually via one of the well-established cytogenetics assays recommended by the International Atomic Energy Agency (IAEA) [2] or the international Standards Organization (ISO) [3]: the dicentric chromosome assay (DCA) or the cytokinesis-block micronucleus (CBMN) assay [4–6]. However, at larger scales, manual analysis of blood samples, even using a large cytogenetic network of labs [7, 8] becomes unfeasible and an increase in overall throughput via automation of triage radiation biodosimetry is needed. The CBMN assay is the most suitable biodosimetry method for automation [9, 10].

While custom robotic systems have been built by us [11] and others [12] for radiation biodosimetry, robotic systems face reliability issues unless in continuous use. On the other hand, universal biotech high-throughput/high content screening (HTS/HCS) robotic systems [13] can switch between several programs, offering a high degree of flexibility for use for different high-throughput biological assays. These systems are in routine use for different purposes [14] ensuring their reliability if needed to respond to a radiological event.

Previously, we proposed the use of commercial universal biotech robotic systems for automated preparation of blood samples and automated imaging in plates compatible with American national Standards Institute (ANSI) and Society for Laboratory Automation and Screening (SLAS) standards [10, 15, 16]. We called this approach RABiT-II (2nd generation Rapid Automated Biodosimetry Technology). Our driving philosophy being that the protocols we develop should be usable on any HTS/HCS system. Many commercial robotic systems are not fitted with an automated centrifuge as they are designed for assays that do not require centrifugation (for example, preparation and analysis of adherent cell lines). Such systems cannot be exploited for preparation of CBMN assay samples using traditional protocols, which rely heavily on centrifugation [2] for washing of non-adherent cells, thus limiting the number of potential RABiT-II systems. The development of a centrifuge-free automated assay could represent a solution for the use of centrifuge-free robotic systems for preparation of samples for CBMN assay and increase the overall throughout of dose estimate in the case of a large radiological event.

Here we describe the development of an accelerated CBMN assay protocol for sample preparation on a commercial robotic system that does not have a centrifuge. As a starting point we used the automated accelerated CBMN assay with a reduced cell culture time (54 h vs the traditional 72 h) [16] we have previously implemented on a cell::explorer system (PerkinElmer, Waltham MA) that has an integrated, automated centrifuge. In that assay we were able to reduce the time to answer of the conventional CBMN assay by 16 h.

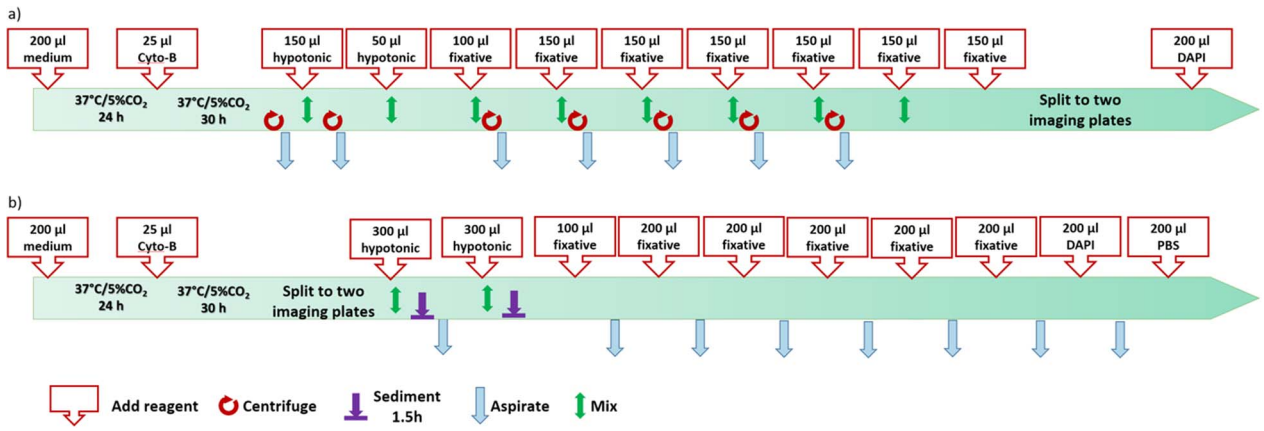

Fig. 1. Overall workflow of the a) accelerated and b) centrifuge-free accelerated CBMN assays developed for the RABiT II system. Dispense: adding the required amount of reagent. Mix: aspire and dispense 200 µL five times. Centrifuge: 1200 rpm/2 min. Aspire: aspire all liquid from about 1 mm above plate bottom—200 µL, Sediment: leave plate on bench for 1.5 h.

MATERIALS AND METHODS

Blood sample collection and irradiation

Blood samples (2 mL) were collected into heparinized vacutainer tubes (BD, Franklin Lakes, NJ) from four healthy volunteers following informed consent (IRB protocol #AAAF2671). A total of 24 aliquots of human blood (20 µL) from each donor were pipetted into 1 mL 2D-barcoded tubes (Matrix Storage Tubes; Thermo Fisher Scientific Inc., Waltham, MA), placed into ANSI/SLAS microplate format compatible 96-tube racks (8 × 12) and covered with the supplied top (Thermo Fisher Scientific). The covered tubes in racks were transported to a Gammacell 40 ^{137}Cs irradiator (Atomic Energy of Canada Ltd., Mississauga, Canada). Blood samples were exposed to 0 (control), 1.0, 2.0, 3.0, 4.0 or 5.0 Gy of γ-rays at a dose rate of 0.70 Gy/min.

RABiT II system

A centrifuge-free RABiT-II system was used for automated sample processing after culturing. The system consists of two parts: the first has an automated liquid-handling system (JANUS, PerkinElmer) with an integrated automated microplate handler (Twister3, Caliper Life Sciences, Waltham, MA) and was used for sample preparation without a centrifuge; the second part, an IN Cell Analyzer 2000 automated imager (GE Healthcare, Chicago, IL), was used for sample imaging.

Automated centrifuge-free sample processing

The overall workflow of the assay, as compared with the assay described in [16] is shown in Fig. 1. The beginning of the assay is the same.

All 96 blood samples (4 sets from each donor) were collected into one 96-tube rack and 200 µL of PB-MAX^{TM} karyotyping medium (Thermo Fisher Scientific) was added to each 20 µL blood sample. The rack of tubes containing blood and PB-MAX^{TM} karyotyping medium was placed into the incubator at 37°C under a 5% CO_2 atmosphere. After 24 h of incubation, cytochalasin-B (Sigma-Aldrich, St. Louis, MO) was added to the cultures at a final concentration of 6 µg/mL, to block cytokinesis of proliferating lymphocytes, and cells were cultured for an additional 30 h (total incubation time 54 h).

After completion of cell culturing, instead of performing hypotonic treatment and fixation in the tubes, all the samples from one set of 96 tubes were divided into two equal parts by transferring 100 µL of each tube to two standard-height glass-bottom 96 square-wells plates (630 µL well volume; Brooks Automation, Chelmsford, MA) preloaded with 300 µL of hypotonic solution (0.075 M potassium chloride). After 1.5 h for sedimentation of cells, the liquid was aspirated leaving ~100 µL, then 300 µL of the same hypotonic solution was added and mixed. After another 1.5 h of cell sedimentation, 100 µL of freshly prepared fixative (3:1 methanol:acetic acid) was added from the top. After 10 min, the fixative (3:1 methanol:acetic acid) was exchanged four times using a 200 µL volume, leaving ~100 µL of liquid in each well after each aspiration step. After that, the fixative was exchanged with 200 µL of fixative with an increased percentage of methanol (10:1 methanol:acetic acid) and the liquid was fully aspirated. The imaging plates were kept for 30 min for complete evaporation of the residual fixative. For DNA staining, 200 µL of PBS containing 1.5 µg/mL DAPI (4′,6-diamidino-2-phenylindole; Thermo Fisher Scientific) was added for 30 min and exchanged with PBS. All reagents were stored on the deck of the automated liquid-handling system at room temperature.

Automated imaging and data analysis

Glass-bottom microplates with fixed and stained samples were scanned on an IN Cell Analyzer 2000. TIFF-images were acquired through a 20x lens with CCD camera (2048 × 2048 pixel resolution) using 350/50 nm excitation and 455/58 nm emission at an exposure time of 300 ms with laser autofocusing. Full well-image acquisition produced 81 images for each 60 mm^2 well.

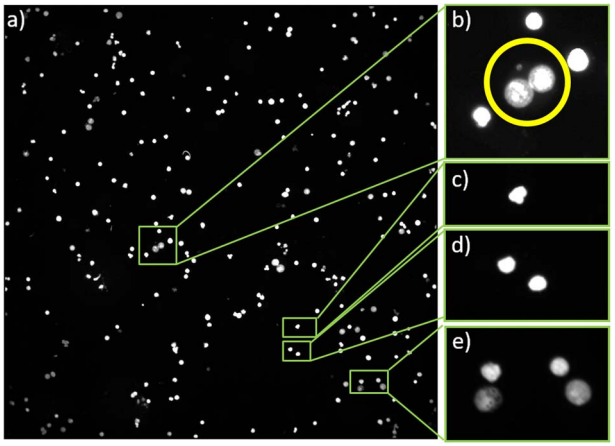

Fig. 2. DAPI-stained image of a blood sample irradiated to 4 Gy and analyzed using the centrifuge-free RABiT-II accelerated CBMN assay. a) One field imaged at 20x. b) Magnified view of a binucleated cell containing a micronucleus and surrounded by three undivided nuclei. c) Nucleus rejected due to improper shape. d) "Binucleate" cell rejected due to too large distance between nuclei. e) Two "binucleate" cells rejected due discrepancy in size/brightness.

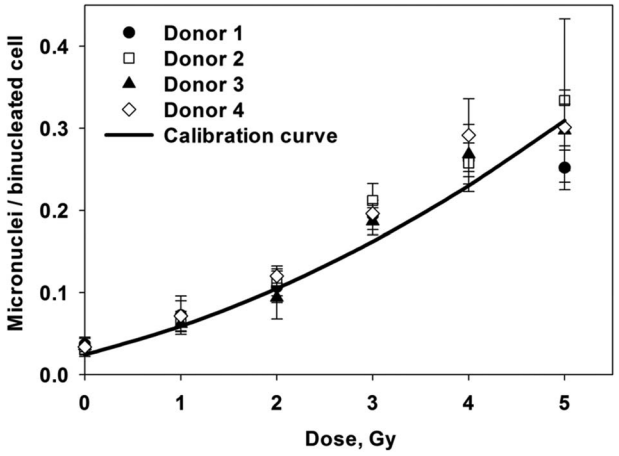

Fig. 3. Radiation dose–response for four healthy donors obtained using the centrifuge-free RABiT-II accelerated CBMN assay. Error bars correspond to standard deviation of four identical samples per donor, processed on the same plate. The solid line is the calibration curve obtained using our previously published assay (Fig. 3 of [16]).

Images were analyzed using the Developer Toolbox 1.9 software (GE Healthcare). Round objects of >0.5 μm granule size (the length of the smallest cross-section through the smallest granule) were identified as micronuclei using the vesicle segmentation module, and round objects of >70 μm^2 were identified as nuclei using the nuclear segmentation module. The data for these identified objects were exported into Microsoft Excel 2010 for further data analysis. Binucleated cells were identified with the following criterion: two nuclei, each with an area >70 μm^2, similar object characteristics (brightness and area are within 5% and 20%, respectively), and with the distance between them less than three times their average radius. A micronucleus was scored if the distance from its center to the midpoint between the centers of two nuclei in the associated binucleated (BN) cell was <13 μm, and also if the area of the micronucleus was less than one-third of the average area of the two nuclei in the associated BN cell.

RESULTS

A typical image showing one field of view in an irradiated human blood sample, processed using the centrifuge-free RABiT-II assay with 54 h of cell incubation, is shown in Fig. 2. Only nucleated cells were observed, red blood cells were eliminated during the hypotonic treatment and fixation.

The number of detected BN cells in the analyzed samples varied, with a maximum of 953 and an average of 419 in control samples and a minimum of 73 BN cells and an average of 117 for samples irradiated with the highest dose of 5.0 Gy.

The dose–response curves of micronuclei yields for four healthy donors are shown in Fig. 3. Also shown in the figure is the calibration curve generated using the automated assay with 54 h culture time and utilizing a centrifuge [16].

DISCUSSION

Centrifugation is a traditional technique for sample preparation in human cytogenetics and centrifugation-based methods are recommended for preparation of samples in radiation biological dosimetry [2]. In this paper, we show that appropriate samples for the CBMN assay can be obtained by using an automated HTS/HCS robotic system without a centrifuge. This approach is based on the substitution of multiple centrifugation steps for sample preparation after whole blood culturing with only two steps of gravity sedimentation of cells in standard-height imaging microplates and fast multiple washes after adherence of mononuclear cells to the glass bottom of microplate wells.

Unlike our standard assay [10, 16], we performed fixation in the imaging plates, rather than in the collection tubes. This resulted in a decreased sample preparation time and a smaller required number of washes (two compared with six, Fig. 1). This is possible as unfixed lymphocytes bind better to the glass bottom of the plate than fixed ones, allowing more vigorous aspiration.

The most critical step in the developing of the centrifuge-free protocol was to attach non-adherent lymphocytes to the bottom of wells. For this purpose we used low-speed dispensing and aspiration of liquid before the complete attachment of cells following the last aspiration of fixative and its evaporation. We used the sedimentation of cells twice instead of once before addition of fixative in order to decrease the amount of debris remaining on the glass bottom of microplate wells, before addition of fixative, to an acceptable level for further image analysis. This also served to remove red blood cells from the preparation, without requiring a ficol gradient which is very hard to automate [19].

After the second sedimentation of cells, fixative is added to the sample to adhere cells to the glass surface. By changing the standard fixative (3:1 methanol:acetic acid) the purity of samples increases with each step. For the last washing step with fixative solution, the increased concentration of methanol (10:1 methanol:acetic acid) was used to increase the quality of cell preparation for image analysis, particularly for improving the separation of micronuclei from main nuclei in BN cells. Following complete evaporation, DAPI solution is used to stain the nuclei content of cells and is changed to PBS after staining in order to increase the contrast of images, as larger debris remains after this procedure as compared with preparation of samples using centrifugation steps.

The quality of CBMN assay samples prepared using the developed centrifuge-free RABiT-II approach (Fig. 2) allows the reproduction of typical dose–response curves using automated image analysis (Fig. 3). These are our first results of the centrifuge-free RABiT-II CBMN assay. As can be seen the results match those of the standard, automated assay [17] rather well. It should be noted that the sedimentation is not as efficient as centrifugation in pelleting the cells and thus cell yields are 5–10-fold lower in the centrifuge-free assay compared with the accelerated one [16]. The developed protocol can be further modified to improve the quality of cells for image analysis. For example, a cell purification step using magnet-beads can be introduced or with the use of antibody capturing of different primary human lymphocytes, as has been demonstrated for a CBMN assay on a microarray platform [18].

The time of sample preparation after culturing using the centrifuge-free robotic system was about 4 h for 96 samples including two gravity sedimentation steps of 1.5 h each. The use of these two sedimentation steps instead of centrifugation steps certainly increases the time of the CBMN assay, but keeps the total assay time within the same 3 days as in the case of the accelerated centrifuge-based RABiT-II CBMN assay [16], including cell culturing, image and data analysis. However, the introduction of such a centrifuge-free approach and use of the corresponding RABiT-II systems in different laboratories and companies has the potential to significantly increase the overall throughput of sample preparation.

Moreover, our protocols for RABiT-II cell harvesting with the use of a centrifuge [10, 16, 19] as well as the centrifuge-free RABiT-II approach described in this paper were developed for keeping all reagents and plates at room temperature so that more workstations without automated heaters/coolers could be used for CBMN assay sample preparation. Thus, almost any ANSI/SLAC-format compatible robotic cell handling system meeting the minimum requirements of the centrifuge-free RABiT-II approach can be used for sample preparation with the use of the corresponding RABiT-II systems, with the potential to increase the capacity for biodosimetry response during a large-scale radiological/nuclear event.

CONCLUSION

The results of this work demonstrate that the accelerated CBMN assay can be automated in a high-throughput format by using centrifuge-free commercial HTS/HCS robotic systems designed for running biological assays in standardized multiwell plates. The usage of centrifuge-free biotech robotics such as RABIT-II systems in multiple laboratories could considerably increase the total throughput of dose estimations in emergency triage radiation biodosimetry.

ACKNOWLEDGMENT

This study was approved by the Columbia Institutional Review Board, protocol #AAAF2671.

CONFLICT OF INTEREST

The authors report no conflict of interest.

FUNDING

This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health [grant number U19 AI067773, the Center for High-Throughput Minimally-Invasive Radiation Biodosimetry]. The content is solely the responsibility of the authors and does not necessarily represent the official views of National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

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